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ANNEX 8

Purification and Properties of a D-Galactose/*N*-Acetyl-D-galactosamine-Specific Lectin from *Erythrina cristagalli*

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The lectin from the seeds of *Erythrina cristagalli* has been isolated in high yield (75%) and homogeneous form by affinity chromatography on a column of D-galactose-derivatised Sepharose. It is a glycoprotein with a molecular weight of 56800 ± 900 and $s_{20,w} = 3.9$ S, composed of two subunits (apparent molecular weights of 28000 and 26000 respectively) both of which are glycosylated. The total carbohydrate content is 4.5% and it is comprised of mannose, *N*-acetylglucosamine, fucose and xylose in amounts corresponding to 7, 4, 2 and 2 mol/56800 Da respectively. The amino acid composition of the lectin is characterised by a high content of acidic and hydroxy amino acids, low content of methionine and absence of cysteine. Valine is the only N-terminal amino acid detected. The lectin is a metalloprotein in that it contains 0.093% Mn and 0.13% Ca (1 mol and 1.9 mol/56800 Da respectively), both of which are tightly bound to the protein.

E. cristagalli lectin agglutinates untreated human erythrocytes of all blood types, as well as rabbit erythrocytes, at a concentration of 5–10 μg/ml. It is mitogenic for human peripheral blood T lymphocytes at an optimal concentration of about 100 μg/ml, but is not mitogenic for mouse thymocytes or splenocytes.

D-Galactose and various D-galactosides inhibit the hemagglutinating activity of the lectin. *N*-Acetyllactosamine is most potent, completely inhibiting four agglutinating units of the lectin at 0.4 mM concentration. Lactose, *N*-acetyl-D-galactosamine and D-galactose are 5, 16 and 35 times less active respectively. Lactose specifically perturbs the ultraviolet spectrum of the lectin in the aromatic region. The difference spectrum obtained upon binding of the disaccharide to the lectin shows maxima at 291 nm and 282–284 nm, indicating a change in the environment of tryptophan residues of the protein upon binding of sugar.

During the last decade there has been a remarkable increase of interest in lectins. Close to 100 lectins have been purified and characterised, and some of them were found to possess unusual structural features (for recent reviews see [1,2]). The sugar specificity of many lectins, and their interactions with different cells, have been investigated. As a result of these studies it became clear that lectins with identical specificity with respect to monosaccharides differ in their interactions with oligosaccharide moieties of glycoconjugates, and therefore also with cells. For example, of all known galactose[1]-specific lectins, only peanut agglutinin distinguishes between mature and immature human and murine thymocytes, while among *N*-acetylgalactosamine-specific lectins only soybean agglutinin interacts selectively with murine B splenocytes and not with T cells [3]. It is therefore important to isolate as many lectins as possible and to study their sugar specificity in detail.

In this paper we describe the isolation of a galactose/*N*-acetylgalactosamine-specific lectin from the seeds of *Erythrina cristagalli* by affinity chromatography on galactose-derivatised Sepharose. The lectin has been studied with respect to its structure, composition, biological activities and sugar specificity. Its properties are compared with those of the recently purified lectins from *Erythrina indica* [4] and *Erythrina corallodendrum* [5].

[1] All sugars were of the D-configuration unless otherwise stated.

MATERIALS AND METHODS

Materials

Divinylsulphone was a product of Polyscience (Warrington, PA), Sepharose 6B was from Pharmacia (Uppsala) and concanavalin A–Sepharose was from Miles-Yeda (Rehovot). Neuraminidase from *Vibrio cholerae* (1 U/ml) was purchased from Behringwerke (Marburg): 1 unit is defined as the amount of enzyme that liberates 1 μmol sialic acid from human α_1 acid glycoprotein in 1 min at 37°C and pH 5.5. Trypsin (2 × crystallised), human transferrin and bovine serum albumin were from Sigma (St Louis), ovalbumin and lysozyme from Worthington (New Jersey), and fetuin from Gibco (Grand Island, NY). *N*-Acetyllactosamine was a synthetic product [6] kindly supplied by Dr A. Veyrières (Université de Paris-Sud). All other sugars were commercial products of highest purity available and all other reagents were of analytical grade. Soybean agglutinin was prepared as described [7] except that acid-treated Sepharose [8] was used as adsorbent for affinity chromatography. *Erythrina cristagalli* seeds were collected by one of us (J. L. I.) in Uruguay. Slightly outdated human blood was obtained from Kaplan Hospital, Rehovot, and rabbit, sheep and mouse blood was drawn from animals supplied by the Animal Breeding Centre at the Weizmann Institute. Asialofetuin was prepared by hydrolysis of fetuin (20 mg/ml, 0.1 M HCl, 80°C for 1 h) and dialysis of the hydrolysate against phosphate-buffered saline, P_i/NaCl (0.05 M potassium/sodium phosphate buffer, pH 7.2, in 0.9%

NaCl). To obtain neuraminidase-treated cells a 20% suspension of erythrocytes in P_i/NaCl was treated with 5 mU enzyme/ml for 1 h at 37 °C and the cells washed three or four times in P_i/NaCl. Trypsinization of erythrocytes was carried out on a 4% cell suspension in P_i/NaCl using 100 μg enzyme/ml, and the cells were washed as above.

Preparation of Affinity Column

Galactose-derivatised Sepharose 6B was prepared by the divinylsulphone method of Porath and Ersson [9]. Packed Sepharose 6B (100 g wet weight) was suspended in 100 ml 0.5 M sodium carbonate buffer, pH 11, and 10 ml divinylsulphone was added. The suspension was kept at room temperature for 70 min with slow stirring and the activated gel was thoroughly washed on a glass filter with distilled water. It was then suspended in 100 ml of a 10% solution (w/v) of galactose in the carbonate buffer and left overnight in the cold room (4–6 °C). The resulting product was washed on a glass filter with 1 l carbonate buffer followed by 2 l water and suspended in P_i/NaCl.

Protein Estimation

The method of Lowry et al. [10] was routinely used with bovine serum albumin as standard. For the purified lectin, concentration was estimated from absorption measurements at 280 nm, using the factor $A^{1\%}_{1\,cm} = 12.4$. This value was obtained by determining the protein concentration by the method of Lowry et al. [10] of a solution of purified lyophilised lectin of known absorbance.

Carbohydrate Determinations

Total neutral carbohydrate content was determined by the phenol/sulphuric acid method [11] using mannose as reference sugar. Individual monosaccharides were determined by gas chromatography as trimethylsilyl derivatives after methanolysis of the lectin in the presence of mannitol as internal standard. A column of 3% SE-30 in the temperature range 140–200 °C at 0.5 °/min was used [12].

Amino Acid Analysis

Amino acid composition was determined on a Dionex D500 amino acid analyser. Hydrolysis was in sealed tubes under nitrogen at 110 °C with 6 M HCl for 22 h, 48 h and 72 h. Values for serine and threonine were obtained by extrapolation to zero-time hydrolysis and the maximal values were taken for the remaining amino acids. Methionine and cysteine were determined as methionine sulphone and cysteic acid, respectively, after oxidation of the protein with performic acid [13]. For estimation of tryptophan, hydrolysis was carried out in 4 M methanesulphonic acid [14]. N-terminal amino acids were determined by Edman degradation on a Beckman model C sequencer with quadrol [*N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine] buffer. Identification of amino acid phenylthiohydantoins was done by high-performance liquid chromatography on a Zorbax 5-μm (Dupont) column.

Metal Content

Analysis of metals was done by atomic absorption on a Perkin-Elmer 306 Spectrophotometer. Salt solutions were prepared with double-distilled water and demetallised on a column of Chelex 100 (Bio-Rad). The lectin solution used for metal determination was dialysed at 4 °C with four changes of each external solution against (a) metal-free saline, (b) 0.1 M EDTA followed by metal-free saline, or (c) 1 M acetic acid, followed by metal-free saline. Any precipitate that formed during dialysis was removed by centrifugation.

Gel Electrophoresis

Discontinuous polyacrylamide gel electrophoresis at pH 8.9 was performed according to Davis [15]. Electrophoresis in the presence of sodium dodecylsulphate was carried out on 10% gels in the discontinuous buffer system of Laemmli [16]. For the determination of the apparent molecular weight of the subunits the gels were calibrated with transferrin (M_r 76000), bovine serum albumin (M_r 68000), ovalbumin (M_r 46000), soybean agglutinin (subunit M_r 30000) and lysozyme (M_r 14000). The gels were stained for protein with Coomassie brilliant blue R 250 and for carbohydrates with the periodate/Schiff reagent [17].

Ultracentrifugal Studies

The sedimentation coefficient was determined in a Spinco model E analytical centrifuge at 52000 rev./min and 20 °C using a 0.57 mg/ml solution of the lectin in P_i/NaCl. The molecular weight was calculated from sedimentation equilibrium data according to Yphantis [18], obtained at 17000 rev./min and a protein concentration of 0.35 mg/ml. The partial specific volume was calculated [19, 20] from the amino acid and sugar composition as 0.724 ml/g.

Affinity Chromatography on Concanavalin-A–Sepharose

Purified *E. cristagalli* lectin (5 mg dissolved in 1 ml P_i/NaCl) was applied to a column (1 × 4 cm) of concanavalin-A–Sepharose, and left in contact with the adsorbent for 15 min at 4 °C. The column was then washed with 10 ml P_i/NaCl and the adsorbed lectin eluted with 0.2 M methyl α-mannoside. Elution was followed by monitoring the absorbance of the effluent at 280 nm.

Hemagglutinating Activity

The hemagglutinating activity of the lectin was assayed by the serial dilution method on microtiter plates, using 50 μl lectin solution and 50 μl of a 4% suspension of erythrocytes. A unit of activity is defined as the lowest concentration of lectin giving visible agglutination. The inhibitory activity of sugars was measured by mixing serial dilutions of the inhibitor with four hemagglutinating units of the lectin before addition of erythrocytes and determining the lowest concentration giving full inhibition of agglutination.

Mitogenic Activity

Human lymphocytes were isolated from freshly drawn peripheral blood by centrifugation on Ficoll-Hypaque [21]. T cells were separated from B cells by rosetting out with sheep erythrocytes [22]. Preparation of mouse thymocytes and splenocytes, treatment of the cells with neuraminidase, culture conditions and measurement of the stimulation of DNA synthesis, were carried out as previously described [23].

Table 1. *Purification of* E. cristagalli *lectin*
The crude extract was obtained from 100 g defatted meal

Step	Volume	Total protein	Specific activity	Total activity	Recovery	Purification
	ml	mg	units/mg	units	%	-fold
Crude extract	800	19000	3	51200	100	1
$(NH_4)_2SO_4$ precipitation						
0–30% fraction	200	2200	0.7	1540	3	
30–60% fraction	200	4620	9	41580	82	3
60–100% fraction	330	5280	0.5	2640	5	
Affinity chromatography	150	180	210	37800	74	70

Purification of E. cristagalli *Lectin*

Unless otherwise stated, all operations were carried out in the cold room. Finely ground seeds were defatted by extraction with petroleum ether and air dried at room temperature. The defatted meal (30 g) was extracted with 300 ml P_i/NaCl for 1 h with stirring, the extract was filtered through cheese-cloth and clarified in a Sorvall centrifuge at 13000 rev./min for 10 min. Ammonium sulphate (17.6 g/100 ml) was added to the supernatant with stirring, and the precipitate was removed by centrifugation as above; more ammonium sulphate (19.8 g/100 ml) was added and the mixture kept overnight. The precipitate was collected by centrifugation, suspended in distilled water (80–90 ml) and dialysed extensively, first against distilled water (2 × 5 l) and finally against P_i/NaCl. Any precipitate that formed during dialysis was removed by centrifugation and the clear supernatant was applied to a column (2 × 15 cm) of galactose-derivatised Sepharose, equilibrated with P_i/NaCl. The column was washed with the same buffer until the absorbance at 280 nm of the effluent was ≤ 0.05 and the bound lectin was eluted with 0.2 M galactose. Elution was followed by monitoring the absorbance at 280 nm; the fractions containing protein were collected, dialysed extensively against distilled water and lyophilised.

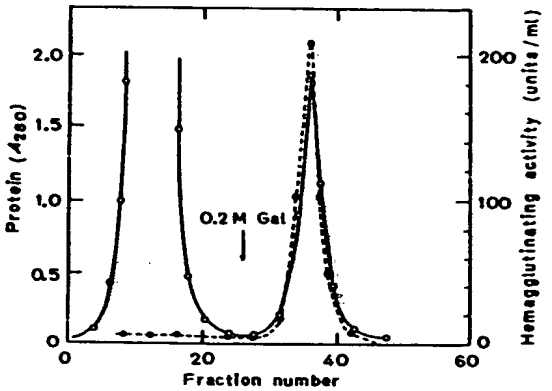

Fig. 1. *Affinity chromatography of* E. cristagalli *lectin on a column of galactose-derivatised Sepharose 6B.* The dialysed ammonium sulphate precipitate obtained from 30 g defatted meal was applied to a 2 × 15-cm column equilibrated with P_i/NaCl. Elution was with 0.6 M galactose. (O——O) A_{280}; (●– – –●) hemagglutinating activity (units/ml)

RESULTS AND DISCUSSION

The presence of hemagglutinating activity in extracts from seeds of different species of *Erythrina* has been known for a long time [24], but only recently have two of the lectins, namely from *Erythrina indica* [4] and from *Erythrina corallodendrum* [5] been purified and characterized.

The *Erythrina cristagalli* lectin described in this paper is eluted from a column of Sepharose-bound galactose as a sharp peak (Fig. 1); neither Sepharose alone, nor acid-treated Sepharose [8] could be used as affinity support, since the lectin did not bind at all to the former and only weakly to the latter. About 50 mg lectin (yield 75%) is obtained from 30 g defatted meal, with a specific hemagglutinating activity of approximately 200 units/mg when determined with untreated human O-type erythrocytes. The purification procedure is summarised in Table 1. As can be seen, the lectin comprises about 1.2% of the soluble proteins of the seeds.

The purified lectin is homogenous by polyacrylamide gel electrophoresis at pH 8.9. It gives a single symmetrical peak in sedimentation velocity and equilibrium experiments, with $s_{20,w} = 3.9$ S and M_r of 56800. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate, two closely migrating bands are obtained, with apparent molecular weights of 28000 and 26000 respectively (Fig. 2). These values are very close to those reported for the *E. indica* lectin ($s_{20,w} = 4.0$ S, molecular weight 66200, and two subunits with molecular weights of 34000 and 30000 [4]). In contrast, the lectin from *E. corallodendrum* has a molecular weight of 110000–120000 [5].

The two bands obtained by polyacrylamide gel electrophoresis of the *E. cristagalli* lectin stained for protein as well as for carbohydrate, indicating that the lectin is composed of two subunits and that both are glycosylated. Homogeneity, as well as the glycoprotein nature of the lectin, was confirmed by chromatography on concanavalin A–Sepharose. The lectin is quantitatively adsorbed to the column and more than 80% of the adsorbed protein is eluted as a sharp peak with 0.2 M methyl α-mannoside (Fig. 3). The combined fractions of the peak contain essentially the total hemagglutinating activity applied to the column. The neutral carbohydrate content of the lectin, as determined by the phenol/sulphuric acid method, is 2.8%. Based on gas chromatography analysis (Table 2), the lectin contains mannose, *N*-acetylglucosamine, fucose and xylose in a molar ratio of 3.5 : 2 : 1 : 1, in amounts corresponding to 7, 4, 2 and 2 mol/56800 Da respectively; the total carbohydrate content was calculated to be 4.5%. A strikingly similar carbohydrate composition has been reported for bromelain, the proteolytic enzyme from pineapple stem [25]. It seems likely that, in analogy to other glycoproteins [26, 27], the carbohydrate is linked to the protein via GlcNAc-GlcNAc-

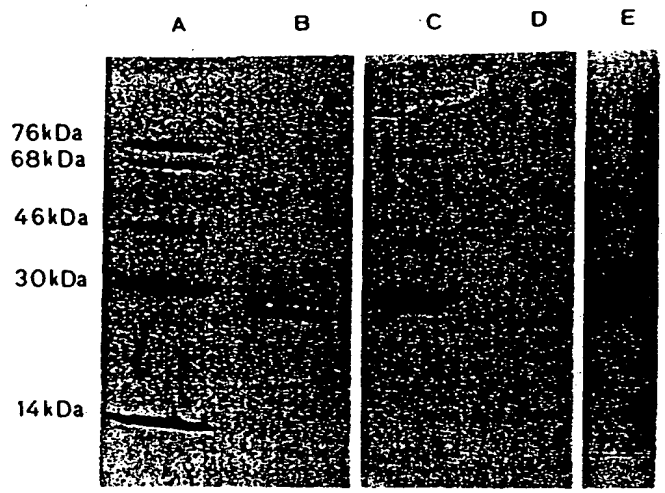

Fig. 2. *Polyacrylamide gel electrophoresis (10%) of purified* E. cristagalli *lectin.* Tracks A–D: in the presence of sodium dodecylsulphate; track E: pH 8.9 without sodium dodecylsulphate. Tracks C and D were stained for carbohydrates with the periodate/Schiff reagent. The markers used (tracks A and C) were transferrin (M_r 76000); bovine serum albumin (M_r 68000); ovalbumin (M_r 46000), soybean agglutinin (subunit M_r 30000); lysozyme (M_r 14000)

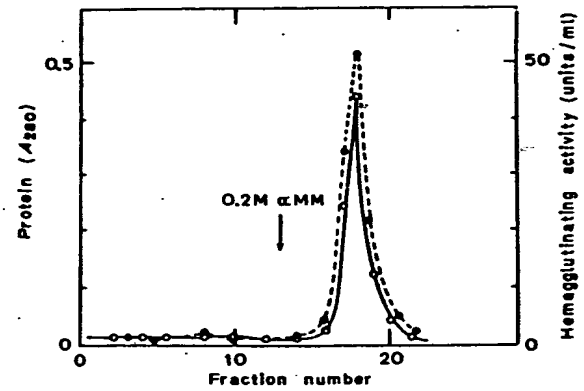

Fig. 3. *Affinity chromatography of purified* E. cristagalli *lectin on a column of concanavalin-A–Sepharose.* 5 mg lectin in 1 ml P_i/NaCl was applied to a 1×4-cm column. Elution was with 0.2 M methyl α-mannoside (α MM). (O—O) A_{280}; (●—●) hemagglutinating activity (units/ml)

Asn. Therefore, there is probably one carbohydrate chain per subunit, in which the mannose residues are attached to the distal *N*-acetylglucosamine in a branched structure.

The amino acid composition of *E. cristagalli* lectin is shown in Table 3. In common with many lectins, it is devoid of cysteine and has a low content of methionine, but is rich in acidic and hydroxy amino acids. Valine is the only N-terminal amino acid detected. The lectin contains 0.093% Mn (1 mol/56800 Da) and 0.13% Ca (1.9 mol/56800 Da). Exhaustive dialysis against 0.1 M EDTA or 1 M acetic acid does not decrease the metal content and hemagglutinating activity of the preparation, although the latter treatment has been shown to lead to the demetallization and loss of biological activity of many lectins [28, 29]. When hemagglutination is carried out in the presence of EDTA at a final concentration of 0.05 M, no effect on the agglutinating activity of the lectin is observed. With the galactose/*N*-acetylgalactosamine-specific lectin from *Sophora japonica*, EDTA at a concentration as low as 0.23 mM completely inhibited four agglutinating doses of the lectin [30]. From the above results, no conclusion can be drawn concerning the importance of the metals for the biological activity of the *E. cristagalli* lectin. It appears, however, that they are tightly bound to the protein.

Table 2. *Carbohydrate composition of* E. cristagalli *lectin*
The monosaccharides were determined by gas chromatography as trimethylsilyl derivatives. The value for *N*-acetylglucosamine is based on the glucosamine content. Results are based on a molecular weight of 56800

Monosaccharide	Composition
	mol/mol
Fucose	1.9
Xylose	2.0
Manpose	7.1
N-Acetylglucosamine	3.8

Table 3. *Amino acid composition of* E. cristagalli *lectin*
Results are based on a molecular weight of 56800 with 4.5% carbohydrate

Amino acid	Composition	
	residues/mol	mol/100 mol
Asp	62	11.5
Thr	43	8.0
Ser	47	8.9
Glu	55	10.2
Pro	39	7.2
Gly	39	7.2
Ala	40	7.4
Half-Cys	0	0
Val	42	7.8
Met	6	1.1
Ile	30	5.6
Leu	37	6.9
Tyr	20	3.7
Phe	28	5.2
His	8	1.5
Lys	20	3.7
Arg	11	2.0
Trp	11	2.0

Table 4. *Agglutination of erythrocytes from various species by* E. cristagalli *lectin*

Origin of erythrocytes	Minimal hemagglutinating dose		
	untreated	trypsinised	neuraminidase-treated
	μg/ml		
Human A type	10		
Human B type	10		
Human O type	5	0.6	1.2
Rabbit	10	0.16	n.d.
Mouse	*	*	30
Sheep	*	*	4

* No agglutination at 1 mg lectin/ml.
n.d. = not determined.

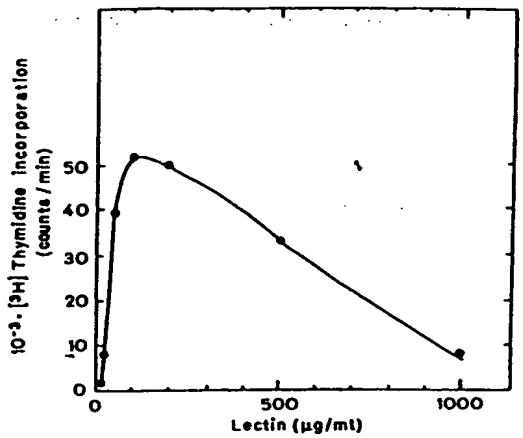

Fig. 4. *Mitogenic stimulation of human peripheral blood lymphocytes by purified* E. cristagalli *lectin.* Cells (2×10^5) were cultured for 48 h with 0.2 ml RPMI 1640 in the presence of varying amounts of lectin. [^{3}H]-Thymidine was added 6 h before harvesting

Table 5. *Mitogenic activity of* E. cristagalli *lectin tested with human peripheral blood T and B lymphocytes*
Assay conditions as described in legend to Fig. 4. Results are expressed as the stimulation index, i.e. ratio of thymidine incorporated in the presence and absence of lectin

Lymphocytes	Stimulation index with the following concn (µg/ml) lectin in assay					
	25	50	100	250	500	1000
Total peripheral blood	13	41	29	6	2	2
T cells	20	67	52	10	3	2
B cells	4	6	9	3	3	1

The lectin agglutinates untreated human erythrocytes of all blood types (with a slight preference for O-type cells), as well as rabbit erythrocytes, at a concentration of 5–10 µg/ml (Table 4). With trypsinised cells about 20-fold lower concentrations of the lectin are required to cause agglutination. Treatment of human erythrocytes with neuraminidase decreases the minimal agglutinating concentration of the lectin by a factor of four. On the other hand, this enzyme has a very strong effect on the susceptibility to agglutination of sheep and mouse erythrocytes: microgram quantities of the lectin are sufficient to agglutinate neuraminidase-treated cells from both animals, while untreated and trypsinised cells are not agglutinated even at 1 mg lectin/ml.

The lectin is mitogenic for untreated (Fig. 4) and desialylated (results not shown) human peripheral blood lymphocytes, at an optimal concentration of about 100 µg/ml for both types of cell. As can be seen from Table 5, the mitogenic activity is directed specifically towards T lymphocytes. The lectin, however, does not stimulate untreated or neuraminidase-treated mouse thymocytes or splenocytes. No information on the mitogenic activity of the *E. indica* lectin is available, but the *E. corallodendrum* lectin is active only with neuraminidase-treated cells.

Of the various carbohydrates tested for inhibition of hemagglutination by *E. cristagalli* lectin, glucose, *N*-acetylglucosamine, mannose, methyl α-mannoside, xylose, L-fucose, L-arabinose and L-rhamnose have no effect up to a concentration of 0.1 M, whereas galactose and all saccharides containing this sugar at the non-reducing end, exhibit various degrees of inhibitory activity (Table 6). *N*-Acetyllactosamine [Gal-β(1-4)GlcNAc] is most potent, completely inhibiting four units of the lectin at 0.4 mM concentration. Lactose and galactose are 5 and 35 times less active respectively. The inhibitory activity of *N*-acetylgalactosamine is between that of lactose and that of galactose. The inhibition studies thus show that the *E. cristagalli* lectin recognises the configuration at the C-4 of the galactopyranoside ring. Replacement of the hydroxyl group at C-2 with an acetamido group increases the inhibitory activity of the sugar; the presence of an amino group greatly decreases this activity. A hydroxymethyl (or methyl) group at C-5 is also important, since L-arabinose is not an inhibitor. The fact that *N*-acetyllactosamine is the best inhibitor and that this disaccharide is five times more active than lactose, strongly suggests that the lectin possesses an extended binding site and that the 2-acetamido group on the penultimate sugar is important for recognition by the lectin.

Table 6. *Inhibition by various sugars of the hemagglutinating activity of* E. cristagalli *lectin with human O erythrocytes*
Other sugars tested, i.e. glucose, *N*-acetylglucosamine, mannose, methyl α-mannoside, xylose, L-fucose, L-arabinose and L-rhamnose were non-inhibitory at concentrations of 0.1 M. The minimal inhibitory concentrations is that required to inhibit completely 4 units of the lectin. The values given are the average of four determinations, starting with different concentrations of the inhibitors. The results are accurate within ± 30%

Inhibitor	Minimal inhibitory concentration
	mM
N-Acetyllactosamine	0.4
Lactose	2.0
p-Nitrophenyl β-galactoside	2.0
p-Nitrophenyl α-galactoside	4.0
N-Acetylgalactosamine	6.5
Methyl α-galactoside	4.8
Methyl β-galactoside	13.5
Galactose	13.5
D-Fucose	18.0
Raffinose	7.2
Galactosamine	20.0
	mg/ml
Asialofetuin	1.56

Although the lectin possesses an extended binding site, galactosides are only slightly better inhibitors than is free galactose. The *p*-nitrophenyl derivatives are not significantly different from methyl galactosides. However, while methyl α-galactoside is a stronger inhibitor than its β anomer, with *p*-nitrophenyl derivatives the β anomer is more potent than the α anomer. Similar behaviour has been found with other galactose/*N*-acetylgalactosamine-specific lectins, e.g. that from *S. japonica* [30], *Wistaria floribunda* [31], and *Psophocarpus tetragonolobus* [32].

Lactose perturbs the ultraviolet spectrum of the lectin in the aromatic region (Fig. 5). The effect is specific, since no change in the spectrum is obtained upon treatment of the lectin with glucose. The difference spectrum obtained upon binding of the disaccharide shows maxima at 291 nm and 282–284 nm, indicating a change in the environment of tryptophan residues upon sugar binding [33].

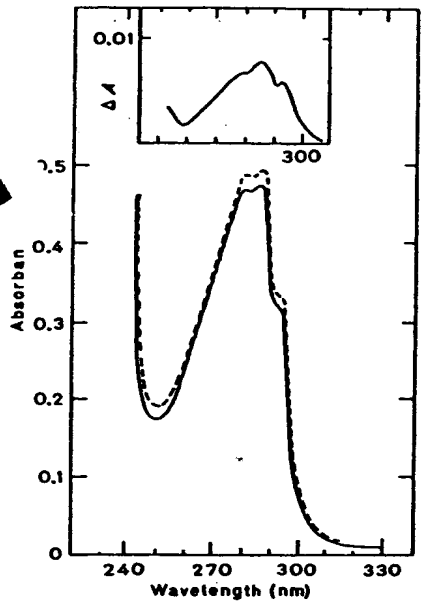

Fig. 5. *Ultraviolet spectrum of* E. cristagalli *lectin (0.4 mg/ml) in the presence of 25 mM lactose (---) and in its absence (——).* Inset: difference spectrum

A comparison of the sugar specificities of the known *Erythrina* lectins reveals many similarities, in particular between those of *E. corallodendrum* and *E. cristagalli* lectins. The lectin from *E. indica* differs from the two other lectins only in that *N*-acetylgalactosamine appears to be four times more efficient an inhibitor than lactose.

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